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Abstract

BACKGROUND – The bacterium *Escherichia coli* is a commonly used host for the production of recombinant protein biopharmaceutical products. One class of such molecules is antibody fragments, typified by the Crohn's disease and rheumatoid arthritis therapy Certolizumab pegol (Cimzia®). Antibody fragments generated in *E. coli* are often directed to the periplasm, so that disulphide bonding can occur and release can be simplified. However, many recombinant protein products are prone to misfolding and mislocalisation. Here, we optimised the production of a Fab fragment, D1.3, and its release from the periplasm of *E. coli* using osmotic shock.

RESULTS – By minimising stress imposed on the bacterial hosts and monitoring Fab, total protein and DNA concentrations of fractions isolated following osmotic release, we successfully targeted the majority of recombinant Fab to the periplasm and were able to rapidly define optimal harvest points. Coupled optimisation of fermentation and release increased the Fab concentration of the periplasmic extract by more than 20-fold.

CONCLUSION – Simultaneous optimisation of fermentation and periplasmic release allowed rapid definition of operational space and generation recombinant protein in a form compatible with downstream processing steps. This methodology could be used for optimisation of the production of a range of periplasmically-targeted recombinant proteins.

Keywords: Recombinant protein production; Fab fragment; Periplasmic release; High Cell Density Culture

INTRODUCTION

Around 30% of protein biopharmaceuticals are generated in bacterial hosts.[1,2] Of these, antibody fragments are a growing class of protein biopharmaceuticals, comprising the antigen-binding regions of full-length human antibodies.[3] The Fab fragment comprises the light chain variable and constant domains (V_L and C_L) and the variable and constant-1 domains of the heavy chain (V_H and C_H1) of the human immunoglobulin, disulphide bonded together. Although more rapidly cleared from circulation than full-length antibodies, antibody fragments allow greater tissue penetration and can be generated in bacterial hosts.[4] The Crohn's disease and rheumatoid arthritis therapy Certolizumab pegol (Cimzia®, UCB) is based on a Fab fragment expressed in *E. coli*.[2]

Such recombinant proteins provide a challenge for *E. coli*, as they require disulphide bonding. Two possible approaches are used to permit this: use of thioredoxin mutant stains with an oxidising cytoplasm (as opposed to its natural reducing state)[5]; or (more commonly) translocation of the recombinant polypeptide chains to the naturally-oxidising periplasm, where the *E. coli* disulphide bond chaperones (Dsc proteins) are located.[6] Generation of an oxidising cytoplasm in *E. coli* can lead to metabolic problems and poor growth, so the latter route is frequently favoured. Theoretically, accumulation of Fab fragments in the periplasm also simplifies downstream processing. Complete cell lysis by mechanical (e.g. high pressure homogenisation) or non-mechanical means[7], required for release of cytoplasmic proteins, is not needed; rather, the outer membrane can be stripped away using osmotic shock[8] or mild heat treatments.[9,10]

The resultant periplasmic extract is thereby already enriched for the recombinant protein of interest, the periplasm containing only around 4 % to 8 % of the natural *E. coli* cellular proteins, making purification simpler.[11] The periplasmic extract is also of lower volume than a whole cell lysate. In addition, periplasmic protease activity is far lower than that in the cytoplasm, reducing proteolysis of the recombinant protein product. In order for periplasmic

release to succeed, bacterial viability and physical integrity must be maintained upon harvest. Metabolic stress in bacteria generating recombinant proteins such as periplasmically-targeted Fab fragments can cause cell lysis during fermentation or fragility leading to lysis during cell harvesting in industrial centrifuges or subsequent processing steps.[12,13,14] This eliminates the advantages of periplasmic expression as cytoplasmic proteins, membrane components and DNA are all released, complicating subsequent purification of the target recombinant protein.

Recent work has focused on minimisation of stress encountered by bacteria generating cytoplasmic recombinant proteins by decreasing growth temperature and inducer concentration.[1,15] This stress-minimising approach results in slower recombinant protein synthesis, but permits correct folding and processing of the resultant recombinant protein. The result is an increase in not only the yield but also the solubility of recombinant protein. Additionally stress minimisation dramatically limits plasmid loss, increases cell viability and improves process robustness. In this study, stress minimisation strategies were used to: (i) increase the yield of a Fab fragment in the periplasm of *E. coli*; and (ii) optimise its release using osmotic shock. Through manipulation of growth temperature, inducer concentration and the point of induction, the Fab fragment could be directed to accumulate in the periplasm, significantly simplifying purification. This approach, simultaneously assessing the effect of stress minimisation on both fermentation and product release, was able to rapidly assess operational space for both upstream and downstream processes, effectively optimising the whole process. We anticipate that this optimisation method could prove an effective strategy to employ during development of new recombinant protein production and release processes.

EXPERIMENTAL

E. coli cultivation

The tetracycline resistant *E. coli* CLD048 used in this study was provided by FUJIFILM Diosynth Biotechnologies (formerly Avecia Biologics) Billingham, UK and expresses the D1.3

anti-Hen Egg White Lysozyme Fab, directing the heavy and light chains into the periplasm.[16] Fermentation inocula were prepared by inoculating 100 mL of Luria Bertani (LB) broth (0.5% w/v yeast extract, 1% w/v tryptone and 1% w/v NaCl) in a conical flask containing 15 $mg \cdot L^{-1}$ tetracycline with a fresh single colony of *E. coli* CLD048 and shaking at 37°C (fermentation 1 only) or 30 °C and 200 rpm for 13 h. Fermentation was conducted in a 5 L Electrolab FerMac 310/60 fermenter (Tewkesbury, UK). The vessel was filled with 3 L of a medium comprising 14 $g \cdot L^{-1}$ $(NH_4)_2SO_4$, 35 $g \cdot L^{-1}$ glycerol, 20 $g \cdot L^{-1}$ Yeast extract , 2 $g \cdot L^{-1}$ KH_2PO_4, 16.5 $g \cdot L^{-1}$ K_2HPO_4, 7.5 $g \cdot L^{-1}$ citric acid and 1.5 $mL \cdot L^{-1}$ H_3PO_4)[17], autoclaved and allowed to cool before addition of 30 mL of 1 M $MgSO_4.7H_2O$ solution, 6 mL of 1 M $CaCl_2.2H_2O$ solution, 0.6 mL of Antifoam AF204, 3 mL of 15 $mg \cdot mL^{-1}$ Tetracycline and 34 mL of trace metal solution (3.36 $g \cdot L^{-1}$ $FeSO_4.7H_2O$, 0.84 $g \cdot L^{-1}$ $ZnSO_4.7H_2O$, 0.51 $g \cdot L^{-1}$ $MnSO_4.H_2O$, 0.25 $g \cdot L^{-1}$ $Na_2MoO_4.2H_2O$, 0.12 $g \cdot L^{-1}$ $CuSO_4.5H_2O$, 0.36 $g \cdot L^{-1}$ H_3BO_3 and 48 $mL \cdot L^{-1}$ concentrated H_3PO_4, in H_2O, filter sterilised). The pH of the medium was adjusted to 7.0 using NH_4OH. Full details of materials used are listed in the supplementary experimental section (supporting materials).

At start-up the agitation and air-flow rates were set at 200 RPM and 1.0 VVM respectively and the pH was adjusted to 7.0 using 10% (v/v) NH_4OH. Thereafter the agitation speed was progressively increased (up to maximum value of 1000 rpm) to sustain the dissolved oxygen tension (DOT) at the set point of 30%. Once the carbon source had been depleted, indicated by an increased DOT feeding (with 714 $g \cdot L^{-1}$ glycerol and 30 $mL \cdot L^{-1}$ 1 M $MgSO_4$) was started. The feed profiles employed were constant in the case of fermentations 1 & 2 (at 90 $mL \cdot h^{-1}$) and exponential for the remaining fermentations (3-7) according to the following equation:[18]

$$F = \left(\frac{1}{S}\right) \times \left(\frac{\mu}{Y_{xs}} + m\right) \times X_0 \times e^{\mu t}$$

Where: *F* is the feed rate into the bioreactor ($L \cdot h^{-1}$); X_0 is the biomass at start of feed (g dry cell weight); μ is the specific growth rate (set to 0.3 h^{-1}); *t* is the time (h); S is the glycerol concentration (714 $g \cdot L^{-1}$) in the feed; Y_{xs} is the cell yield on glycerol (616.7 mg biomass g

glycerol^{-1})[19]; and m is the maintenance coefficient for glycerol (3.683 mg glycerol g cell^{-1} h^{-1}).[19]

Osmotic shock separation of periplasmic and cytoplasmic fractions

One millilitre samples of fermentation broth were centrifuged in a microcentrifuge (15800 g_{av}, 120 s). The supernatants (culture broth fractions) were carefully removed, and the cell pellets were resuspended in 'osmotic shock solution 1' (20 mM Tris-HCl buffer, pH 8 supplemented with 2.5 mM EDTA and 20% w/v sucrose). After incubation on ice for 600 s, the treated cells were recovered by recentrifugation (15800 g_{av}, 120 s), and the supernatants (OS1 fractions, containing periplasmic proteins) were pipetted off and retained. Subsequently the pelleted spheroplasts were re-suspended in 1 mL of 'osmotic shock solution 2' (20 mM Tris-HCl pH 8 2.5 mM EDTA) and incubated on ice for a further 600 s, before finally centrifuging the samples (as above), removing and retaining the supernatants (OS2 fraction, containing cytoplasmic proteins). All of the generated fractions were stored at -20 °C prior to be subjecting to further analysis by (SDS-PAGE, protein or DNA assay).

Fractionation of soluble and insoluble proteins from cell pellets

'Bugbuster' reagent (Merck) was employed to separate soluble and insoluble proteins from *E. coli* pellets. Portions (1 mL) of fermentation broth were subjected to centrifugation (15800 g_{av}, 600 s) in a microfuge. The supernatants were removed, and the pellets were resuspended in a calculated volume (i.e. '67 × culture OD_{600}' µL) of 'Bugbuster' reagent. After incubating for 900 s at room temperature, the 'Bugbuster/cell' cocktails were centrifuged (15800 g_{av}, 1200 s) in a refrigerated microcentrifuge (4 °C), and the supernatants (containing soluble proteins) and pellets (containing insoluble proteins) were separated from one another. The supernatants were immediately mixed with equal volumes of Laemmli Sample Buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% w/v SDS, 26.3% w/v glycerol, 0.01% w/v

bromophenol blue and 5% v/v β-mercaptoethanol)[20], whereas the pellets were washed with phosphate buffered saline, resuspended in '67 × culture OD_{600}' µL of Laemmli buffer and boiled for 600 s.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The protein compositions of soluble (10 µL) and insoluble (5 µL) protein samples prepared above were analysed by reducing SDS-PAGE[20] in a Mini-Protean® Tetracell electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA). Prior to electrophoresis, all samples were diluted 1:1 with the Laemmli Sample Buffer,[20] boiled at 110°C for 600 s, and then centrifuged (7000 g_{av}, 15 s), before loading into the wells of the of a precast 15% (w/v) polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA). SeeBlue Plus2 Pre-Stained Standard (Invitrogen) was used for estimation of molecular weight of protein bands on the gel. Following electrophoresis, gels were rinsed three times with 100 mL of water and then stained with SimplyBlue Safe Stain (Invitrogen) for 1 h at room temperature with gentle shaking. The stain was discarded and the gel was subsequently washed twice with 100 mL of distilled water for 1 h with gentle shaking.

Western Blotting

Proteins were separated by SDS-PAGE and the gel soaked in protein transfer buffer (3.03 $g \cdot L^{-1}$ Trizma-base, 14.4 $g \cdot L^{-1}$ glycine and 200 $mL \cdot L^{-1}$ methanol) for 30 minutes. Hybond-P PVDF membrane and six layers of thick filter paper (Whatman GB005) were cut to the dimensions of the gel, soaked in methanol for 10 seconds, washed in distilled water for 5 minutes, and equilibrated in protein transfer buffer for 30 minutes. The electroblotting cassette was assembled according to the manufacturer's instructions with three layers of filter paper on each side of the gel and membrane (Mini Trans-Blot electrophoretic Transfer Cell, Bio-Rad, CA, USA). The transfer was carried out initially for 1 h at 100 V with transfer buffer at 4 °C, and was then perpetuated at 30 V overnight at 4 °C. Following transfer, the

membrane was removed from the blotting cassette and rinsed briefly in PBS. The membrane was blocked (3% (w/v) BSA in PBS, 1 hour), rinsed three time (0.1 % (v/v) Tween 20 in PBS, 5 minutes each) and incubated with antibody (anti-human IgG (Fab specific) peroxidase conjugated (Sigma) diluted in PBS 10 000-fold) for 1 h. The blot was washed three times (0.1 % (v/v) Tween 20 in PBS, 5 minutes each) before incubation with 15 mL of TMB membrane peroxidise substrate system (3-C) solution (KPL, Gaithersburg, MD, USA) until desired band intensity was achieved. The reaction was stopped by washing in distilled water. Blots were scanned (Canon Canoscan 9000F) and ImageJ[21] was used to determine the relative quantity of soluble and insoluble D 1.3 protein.

Sandwich ELISA

Each well of a 96-well microtiter plate was coated overnight at 4 °C with 100 µL of 0.1 % (w/v) hen egg white lysozyme (Sigma) in coating buffer (1.59 $g \cdot L^{-1}$ Na_2CO_3 and 2.93 $g \cdot L^{-1}$ $NaHCO_3$). Nonspecific binding was blocked by incubation for 1 hour at 37 °C with 200 µL of 10 $g \cdot L^{-1}$ BSA (Sigma) in PBS. Wells were washed three times with 300 µL of wash buffer (0.1 % (v/v) Tween 20 in PBS) per well and tap dried. 100 µL of standards (Purified Fab D1.3, supplied by Reza Jalalirad) or samples were loaded into each well and incubated at 37 °C for 1 h with shaking. The plate was washed again with wash buffer three times and tap dried. Each well was then loaded with 100 µL of antibody (anti-human IgG (Fab specific) - peroxidase conjugated antibody produced in goat (Sigma), diluted 10 000-fold in the block buffer) and incubated at 37 °C for 1 h. The plate was washed again three times tap dried, before 100 µL of peroxidase substrate (TMB microwell peroxidase substrate (2-C), KPL, Gaithersburg, MD, USA) was added to each well. The reaction was stopped after 10 minutes by adding 100 µL of 1 M H_3PO_4 to each well, and the plate was read at 450 nm using a microplate reader (Promega Glomax-Multi detection system, Turner BioSystems Inc., Sunnyvale, CA, USA).

DNA and protein assays

DNA concentrations in samples were determined using a diphenylamine (DPA) based colorimetric assay[22] adapted for use in a microwell plate format. Standards (1 $mg \cdot mL^{-1}$ sonicated calf thymus DNA (3-20 kb in size) in 10 mM Tris-HCl, 1 mM EDTA, pH 8) and samples were dispensed into the wells of a 96 well plate and each well made up to 175 µL with 10 mM Tris-HCl, 1 mM EDTA, pH 8. To each well was added 5 µL of 2 $mg \cdot mL^{-1}$ BSA in water and 200 µL of 0.4 M perchloric acid (PCA) and the plate incubated at 4 ˚C for 0.5 h. Plates were centrifuged (15800 g_{av}, 1200 s), the supernatant discarded, 250 µL of 1 M PCA added to each pellet, vortexed, transferred to a microfuge tube and incubated at 70°C for 0.5 h. Once cooled to room temperature, 500 µL of freshly prepared chromogenic reagent (1.5 g diphenylamine, 100 mL of glacial acetic acid and 1.5 mL of concentrated H_2SO_4, with the addition of 0.5 mL ethanol mixed with 25 mL of water immediately prior to use) was added to each tube. After overnight incubation and subsequent centrifugation (15 800 g_{av}, 600 s) the absorbance of the supernatant was measured in a spectrophotometer at a wavelength of 600 nm. The amount of total protein present in the samples was measured using a bicinchoninic acid assay (BCA assay kit, Thermo Scientific, Rockford, IL, USA) employing bovine serum albumin (BSA) as a standard.

RESULTS AND DISCUSSION

Fed-batch fermentation using conventional approaches

In order assess the impact of altering fermentation conditions on product capture from the *E. coli* periplasm, an integrated approach was used to analyse Fab production. Following collection of samples, bacteria were harvested by centrifugation; the supernatant comprised the culture broth fraction. Bacteria were subjected to osmotic treatment, generating two

further fractions: OS1, comprising the periplasm; and OS2, containing the cytoplasmic proteins. Concentrations of Fab D1.3 product in each fraction were measured by ELISA.

The initial fed-batch fermentation conditions were taken from Hodgson *et al*.[16] The temperature was set at 37 °C and glycerol was fed when the carbon source in the initial batch medium was depleted (8 h after inoculation, determined by a decrease in OD_{600} reading). Fab production was induced after 10 h growth at an OD_{600} of around 61 with 100 μM IPTG (Fig. 1a).

Upon induction, growth slowed and reached a peak biomass (OD_{600} = 70) 6 h after induction, followed by a decrease in biomass concentration thought to be due to cell lysis. ELISA data (Fig. 1b) revealed that very low quantities of Fab D1.3 were generated during the fermentation, up a maximum of around 1.4 $mg{\cdot}L^{-1}$. Although the majority of this Fab was present in the periplasmic (OS1) fraction, the yield was too low to be commercially viable (a maximum of 1 $mg{\cdot}L^{-1}$), especially when compared to other expression data in the literature (for example, Humphreys & Bowering[10]). In addition, the quantity of Fab D1.3 in the culture broth rapidly increased towards the end of the fermentation, signifying cell lysis.

In order to improve the yield of Fab D1.3, the growth temperature was decreased to 30 °C (Fig. 1c). Other parameters were kept the same as fermentation 1. Growth stopped around 4 h following induction of Fab production at a maximum OD_{600} of 63, and although the total Fab yield was far higher (peaking at >31 $mg{\cdot}L^{-1}$), at 4 h post-induction onwards most of the Fab was present in the culture broth fraction, with only a small quantity in the periplasmic fraction (Fig. 1d).

Since *E. coli* does not naturally secrete recombinant proteins, this is due to release of periplasmic Fab by outer membrane damage. This is problematic for industrial processes since periplasmic release requires bacteria to be physically intact to permit selective release of periplasmically-targeted recombinant proteins and prevent release of cytoplasmic proteins during centrifugal cell harvesting or osmotic release procedures.[12,14] If bacteria are physically weakened, for example by RPP-induced stress, then far more protein is released,

complicating DSP; the advantages of periplasmic targeting and release are minimal as compared to cytoplasmic expression, lysis and purification from whole cell lysate. In addition, cell lysis dramatically increases the conductivity of the culture broth, making chromatographic separation difficult without extensive feedstock conditioning steps. Therefore the Fab D1.3 production process was optimised to maximise Fab yield and purity in the periplasmic (OS1) fraction. This provides a useful indication of both Fab productivity and the physical state of the bacteria, in particular the ability of the outer membrane to contain periplasmic proteins. Ideally, a large quantity of Fab should accumulate in the periplasm, contained within a robust outer membrane, which can be readily released by osmotic shock. In addition, fermentations were optimised to prolong the growth of cultures following induction.

Stress minimisation

Stress-minimisation[15,23] was applied to Fab D1.3 production in order to improve protein yield and direct Fab to the periplasm. As well as previously observed benefits of this approach (higher yield and better folding of cytoplasmically-targetted recombinant protein), we wanted to investigate whether stress minimisation also allowed correct targeting into the periplasm and increased physical integrity of bacteria.

Initial scoping studies in shake flasks at 100 mL scale generated promising results (data not shown), so the approach was implemented in bioreactor cultures. The first stress-minimising bioreactor growth employed three principles of stress-minimised RPP[15,23]: Growth at 25 °C, which decreases both the rate of cell division and thus nutrient requirements for biomass production and protein synthesis rates; induction with a low quantity of the inducer IPTG (20 μM), which decreases the rate of Fab production; and early induction (at an OD_{600} of around 1), permitting concurrent Fab production and biomass generation. In addition to ELISA quantification of Fab D1.3 in the osmotic shock fractions, total protein concentrations were

measured along with the concentration of DNA in the culture broth as an indicator of cell lysis.

Unlike previous fermentations (Fig. 1), bacteria continued growing for a significant time following induction (reaching an OD_{600} of 47 after 31 h; Fig. 2a), signifying that growth and RPP were balanced. The lower growth temperature and thus growth rate also ensured that dissolved oxygen was maintained throughout. Total Fab D1.3 concentration increased steadily over time after induction, reaching around 11 $mg \cdot L^{-1}$ after 27.5 h post-induction (Fig. 2b). As before, a small quantity of Fab (<1 $mg \cdot L^{-1}$) was detected in the cytoplasmic fraction, corresponding to newly-synthesised Fab protein before transport to the periplasm. Periplasmic Fab concentration steadily increased, although the culture broth Fab concentration also increased, indicating cell lysis; this was confirmed by increased DNA concentrations present in the culture broth (Fig. 2c). Total protein concentration of the periplasmic fraction was between 1-1.6 $g \cdot L^{-1}$, resulting in a maximum Fab purity of 0.8 % at 22.5 h post-induction.

This fermentation protocol was repeated with induction at an OD_{600} of around 20 (Fig. 2d); this resulted in a higher final OD_{600} of >80. However, total Fab concentrations were not significantly higher than in cultures induced at a lower OD_{600} (Fig. 2e), and far more of the Fab was contained within the cytoplasmic fraction, resulting in a lower periplasmic Fab yield. The total protein concentration of the periplasmic fraction was also lower; this might reflect a difference in the stability of the cell envelope during growth in conditions where induction proceeds at a higher biomass. This is also reflected by the lower culture broth DNA concentration observed in this fermentation (Fig. 2f).

In order to increase the yield of periplasmic Fab, the concentration of inducer IPTG was increased to 100 µM, added at an OD_{600} of around 20 (Fig. 3). This increase in IPTG concentration generated far more Fab; total titres reached above 41 $mg \cdot L^{-1}$. The periplasmic Fab concentration peaked at around 12 $mg \cdot L^{-1}$ at 11 h post-induction (28 h after inoculation). Although an improvement over previous fermentations, the relatively high protein

concentration of the periplasmic extract meant that Fab purity in the periplasmic extract was still only 0.6 %. After 11 h post-induction, most of the Fab was located in the culture broth (up to a maximum of up to 35 $mg \cdot L^{-1}$), indicating extensive cell lysis; this was confirmed by high culture broth DNA concentrations (>300 $mg \cdot L^{-1}$) that increased over the course of the fermentation.

The increase of inducer concentration to 100 µM increased stress during the fermentation; to balance this increased stress and resultant cell lysis, while maintaining a higher Fab titre, the growth temperature was further decreased following induction at an OD_{600} of around 20 to 20 °C (Fig 4a). At the end of the fermentation, total Fab accumulation was again around 40 $mg \cdot L^{-1}$; however, nearly half of the Fab was located in the periplasmic fraction (Fig. 4b). The optimal harvest point for this fermentation was found to be 18.5 h post-induction (36.5 h after inoculation); after this point, the culture broth Fab and DNA concentration both increased, indicating cell lysis (Fig. 4c). At 18 h post-induction, the Fab concentration of the periplasmic extract was 15.8 $mg \cdot L^{-1}$, while total protein concentration was 0.85 $g \cdot L^{-1}$, resulting in a purity of 1.9 % (Table 1).

Finally, the IPTG concentration was increased to 0.2 mM to attempt to increase the yield of Fab D1.3 without detrimentally impacting the harvest window or cell integrity (Fig. 4d). Under these conditions, Fab accumulated in the periplasmic fraction more quickly than when 100 µM IPTG was used to induce RPP; the harvest point can be defined as 8 h post-induction (26 h after inoculation), when 20 $mg \cdot L^{-1}$ Fab D1.3 had accumulated in the periplasmic fraction, representing the majority (86 %) of all generated Fab (Fig. 4e). The purity of the periplasmic fraction at this point was 1.7%, comparable to the previous fermentation. Thus, increasing IPTG concentration effectively brought the time of harvest forward by 10 h when compared to use of 0.1 mM IPTG (Fig. 4a), and boosted Fab titre in the periplasmic fraction by 27 %. At timepoints later than 8 h post-induction, increases in the Fab and DNA concentration in the culture broth indicated cell lysis (Fig. 4f).

As well as ELISA analysis of Fab yields in each subcellular fraction, SDS-PAGE and Western blotting were used to determine the quantity of Fab D1.3 present in soluble and insoluble (inclusion body) forms. Bacterial pellets harvested at the end of each fermentation were fractionated into soluble and insoluble fractions using the Bugbuster® reagent (Table 1). The stress minimisation methods clearly improved the solubility of Fab D1.3 whereas high-stress fermentation conditions drove Fab into the insoluble fraction (Fermentations 3-7 compared to 1 and 2).

This logical method to fermentation optimisation using stress-minimisation is an alternative approach to Design of Experiments (DoE) approaches, where computational tools are used to define a limited number of experiments that rapidly explore multi-parameter experimental space. Future work will compare DoE and logic-based approaches to the optimisation of fermentation and periplasmic release of recombinant proteins.

CONCLUSIONS

We have demonstrated that stress minimisation by decreasing culture temperature and inducer concentration can be used to improve not only recombinant protein yields and folding[15,23] but also correct subcellular targeting and overall cell integrity for subsequent cell harvest and protein release steps.[12,13,14] Careful balancing of increases in inducer concentration and decreases in growth and induction temperature allowed optimisation of Fab D1.3 yield. We have also demonstrated the benefits of optimising periplasmically-targeted recombinant protein production using a coupled fermentation – release approach; optimisation of the fermentation stage only would not generate insights into the processability of the biomass generated and its applicability for subsequent periplasmic release and purification of the resultant recombinant proteins. Future studies will expand upon these findings by using a DoE approach to further optimise coupled fermentation and release of recombinant proteins.

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FIGURE LEGENDS

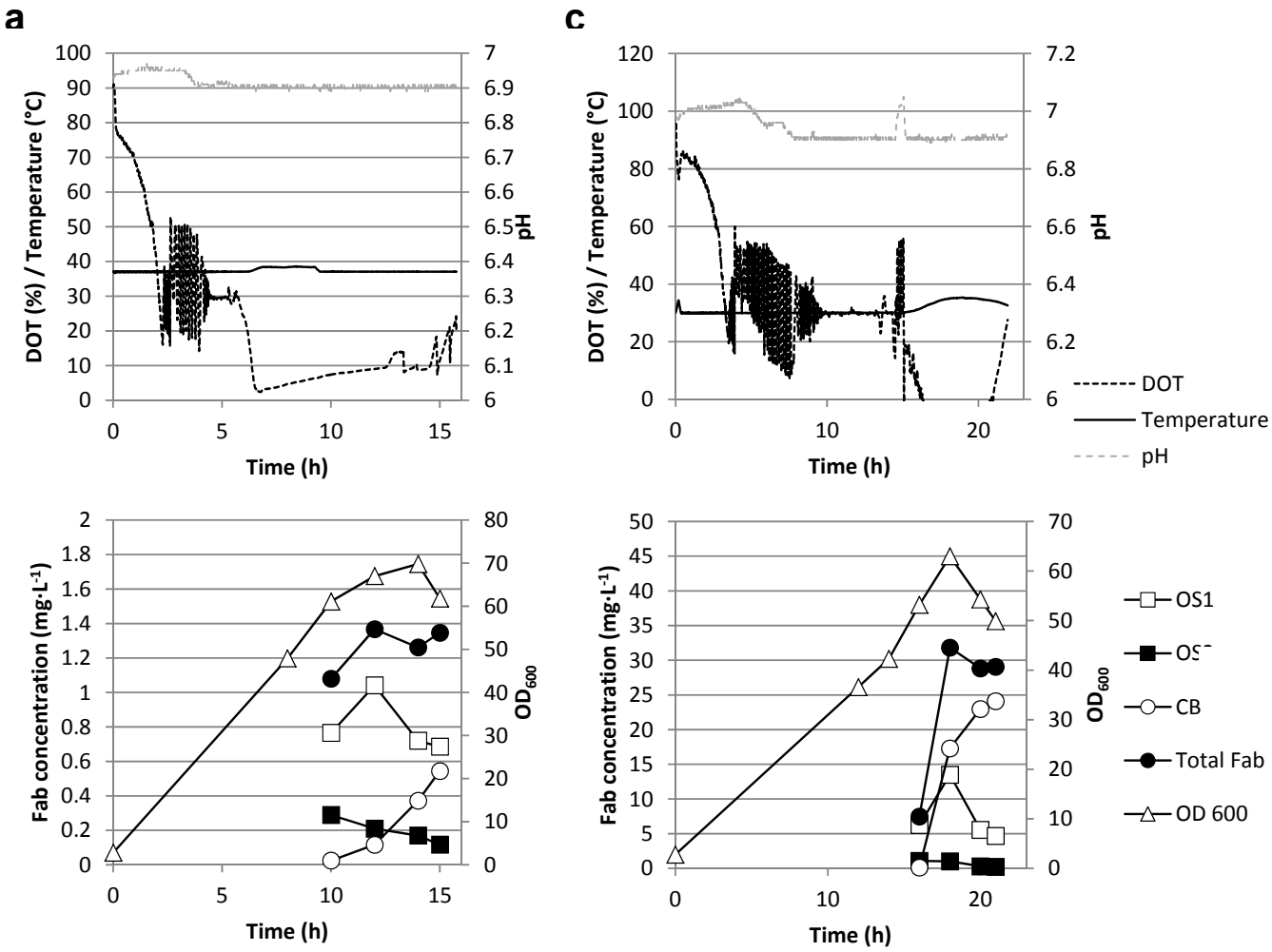


Figure 1. Fermentations 1 and 2 using conventional parameters. For fermentation 1, the conditions were a cultivation temperature of 37°C and induction with 0.1 mM IPTG at an OD_{600} of ~40. **(a)** Online fermentation parameters (Dissolved oxygen%, Temperature and pH). The arrow indicates the point of induction. **(b)** Optical density at 600 nm and concentration of Fab D1.3 measured by ELISA in each fraction. For fermentation 2, the conditions were a cultivation temperature of 30°C and induction at an OD_{600} of ~ 40 with 0.1 mM IPTG. **(c)** Online fermentation parameters (Dissolved oxygen%, Temperature and pH). The arrow indicates the point of induction. **(d)** Optical density at 600 nm and concentration of Fab D1.3 measured by ELISA in each fraction. OS1, periplasmic extract; OS2, cytoplasmic

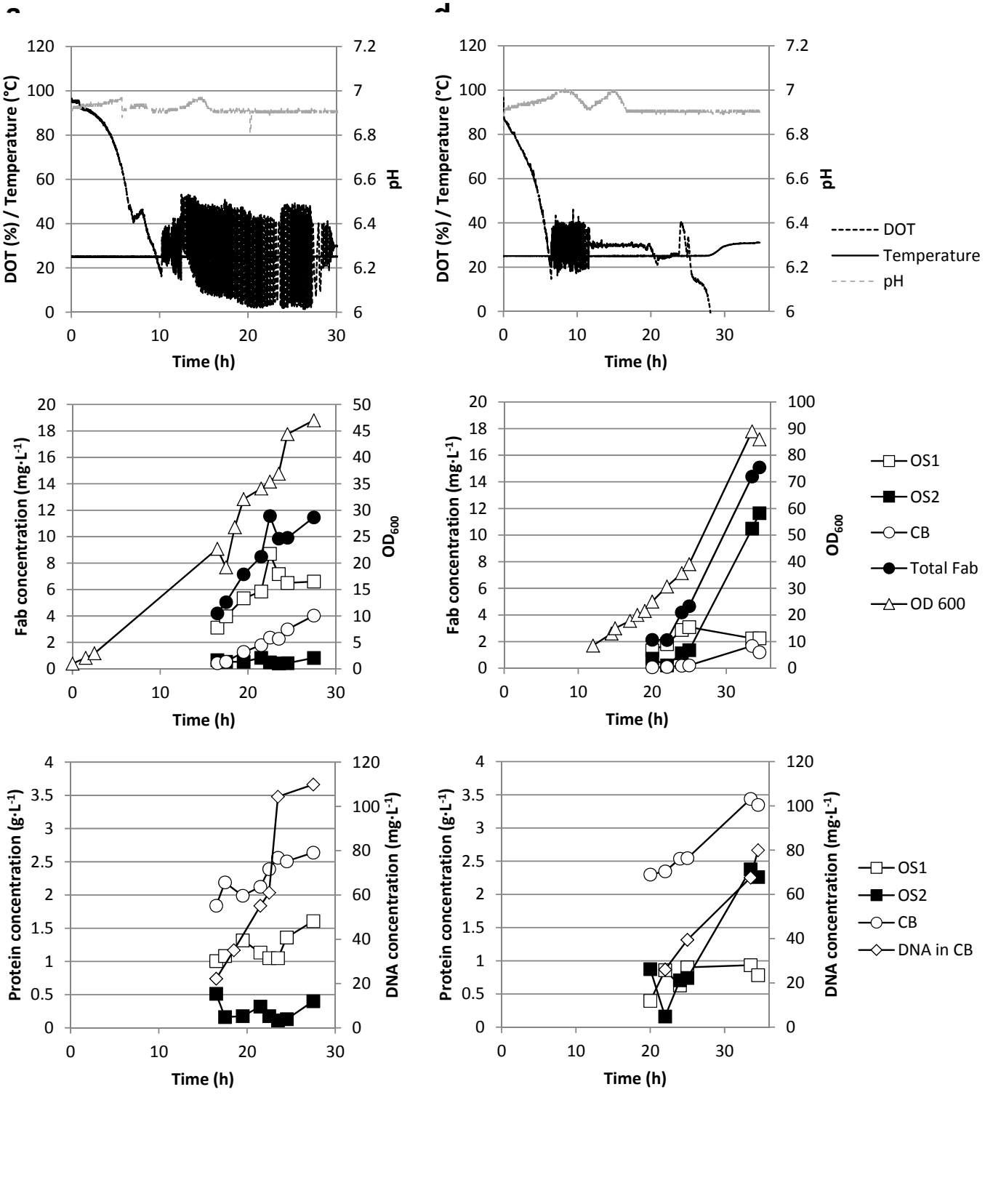
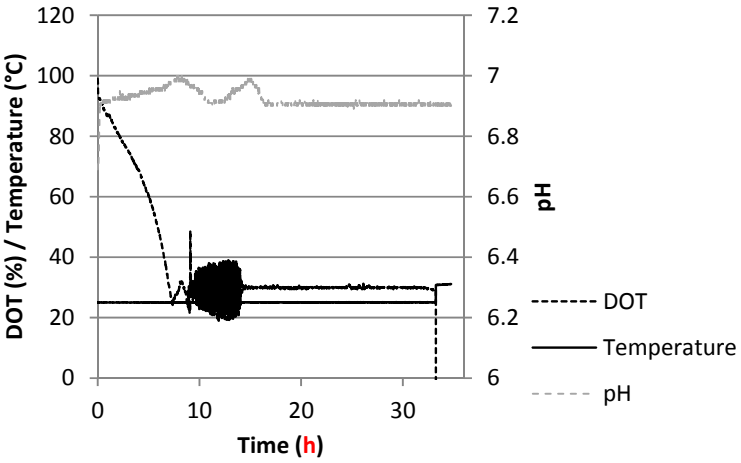
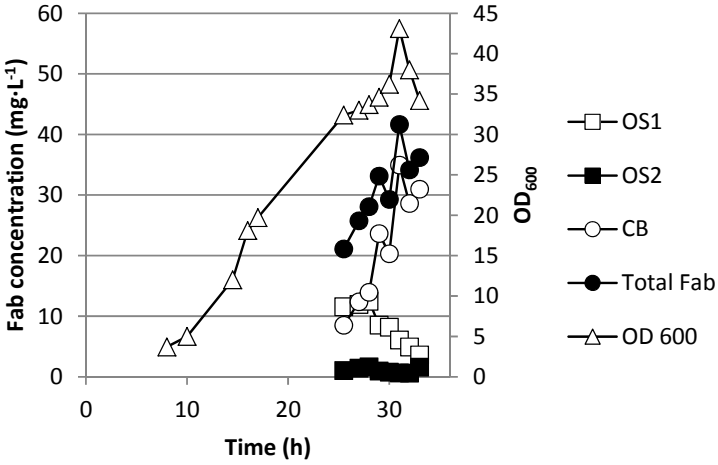


Figure 2. Fermentations 3 and 4 using stress minimisation. For fermentation 3, the temperature was 25°C and RPP was induced with 20 μM IPTG at an OD_{600} ~1. **(a)** Online fermentation parameters (Dissolved oxygen%, Temperature and pH). The arrow indicates the point of induction. **(b)** Optical density at 600 nm and concentration of Fab D1.3 measured by ELISA in each fraction. **(c)** Concentration of total protein in each fraction measured by BCA assay and concentration of DNA in culture broth measured by DPA assay. For fermentation 4, conditions were as fermentation 3 except that induction with 20 μM IPTG occurred at an OD_{600} ~20. **(d)** Online fermentation parameters (Dissolved oxygen %, Temperature and pH). The arrow indicates the point of induction. **(e)** Optical density at 600 nm and concentration of Fab D1.3 measured by ELISA in each fraction. **(f)** Concentration of total protein in each fraction measured by BCA assay and concentration of DNA in culture broth measured by DPA assay. OS1, periplasmic extract; OS2, cytoplasmic extract; CB, culture broth.

a





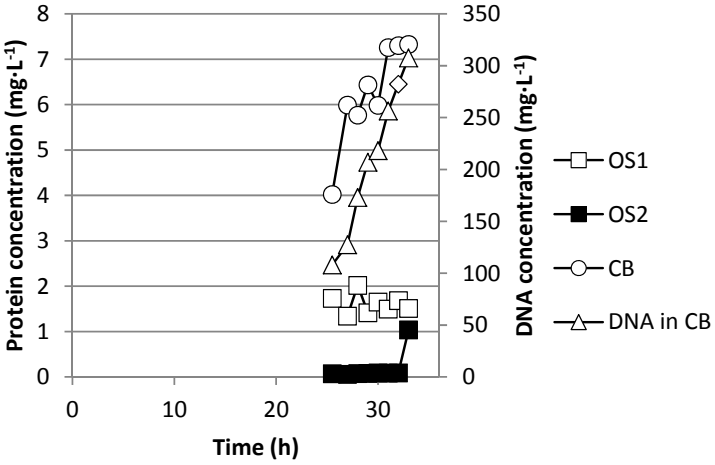


Figure 3. Fermentation 5 using stress minimisation but a higher IPTG concentration. The temperature was 25 °C and RPP was induced with 0.1 mM IPTG at an OD_{600} ~20. **(a)** Online fermentation parameters (Dissolved oxygen%, Temperature and pH). The arrow indicates the point of induction. **(b)** Optical density at 600 nm and concentration of Fab D1.3 measured by ELISA in each fraction. **(c)** Concentration of total protein in each fraction measured by BCA assay and concentration of DNA in culture broth measured by DPA assay. OS1, periplasmic extract; OS2, cytoplasmic extract; CB, culture broth.

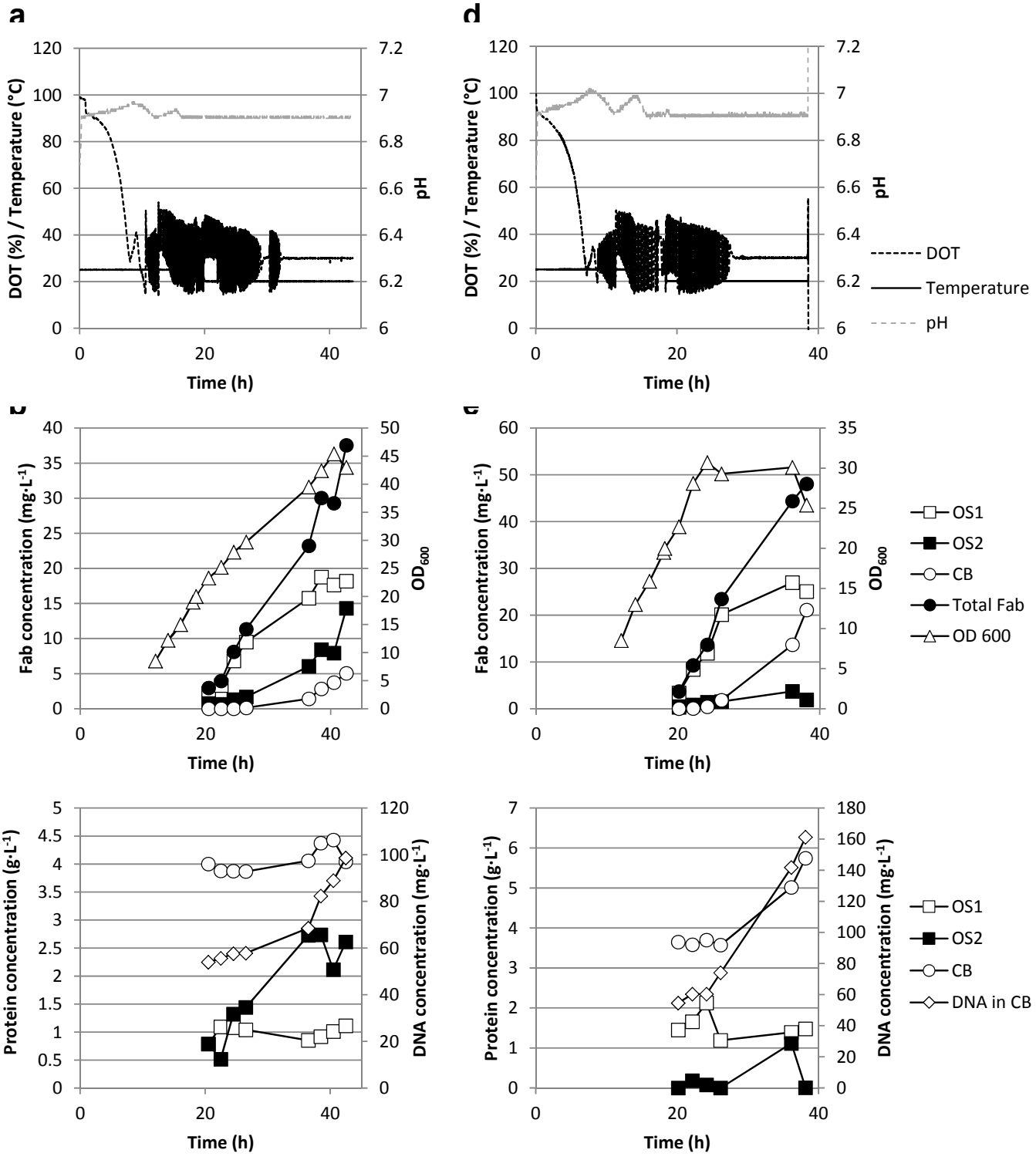


Figure 4. Fermentations 6 and 7 using stress minimisation and an additional temperature decrease. For fermentation 6, the temperature was 25 °C before induction. RPP was induced with 0.1 mM IPTG at an OD_{600} ~20, after which the growth temperature was decreased to 20 °C. **(a)** Online fermentation parameters (Dissolved oxygen%, Temperature and pH). The arrow indicates the point of induction. **(b)** Optical density at 600 nm and concentration of Fab D1.3 measured by ELISA in each fraction. **(c)** Concentration of total protein in each fraction measured by BCA assay and concentration of DNA in culture broth measured by DPA assay. For fermentation 7, conditions were as fermentation 6 except that the IPTG concentration was increased to 0.2 mM. **(d)** Online fermentation parameters (Dissolved oxygen%, Temperature and pH). The arrow indicates the point of induction. **(e)** Optical density at 600 nm and concentration of Fab D1.3 measured by ELISA in each fraction. **(f)** Concentration of total protein in each fraction measured by BCA assay and concentration of DNA in culture broth measured by DPA assay. OS1, periplasmic extract; OS2, cytoplasmic extract; CB, culture broth.

Table 1. Summary of properties of periplasmic extracts from fermentations in this study.

Fermentation No.	Fermentation conditions			Optimal periplasmic (OS1) extract properties				% Fab in soluble fraction[c]
	Temperature (°C)	[IPTG] (μM)	Induction point (OD_{600})	[Fab] ($mg \cdot L^{-1}$)	[Protein] ($mg \cdot L^{-1}$)	Fab purity (%)[a]	Harvest point (h)[b]	
1	37	100	48	1.0	ND	ND	12	25
2	30	100	42	13.5	ND	ND	18	40
3	25	20	1	8.7	1050	0.8	22.5	94
4	25	20	20	3.1	900	0.3	25	74
5	25	100	20	12.5	2020	0.6	28	64
6	25 → 20	100	20	15.8	850	1.9	36.5	77
7	25 → 20	200	20	20	1190	1.7	26	61

a. 100 x [Fab]/[Protein] for OS1 fraction.

b. Time point for OS1 (periplasmic extract) data; relates to optimal harvest point as described in text.

c. Determined by Bugbuster analysis followed by Western blotting and densitometric quantification; samples taken at end of fermentation.

ND: Not determined